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Effects of heat and cadmium exposure on stress-related responses in the liver of female zebrafish: Heat increases cadmium toxicity

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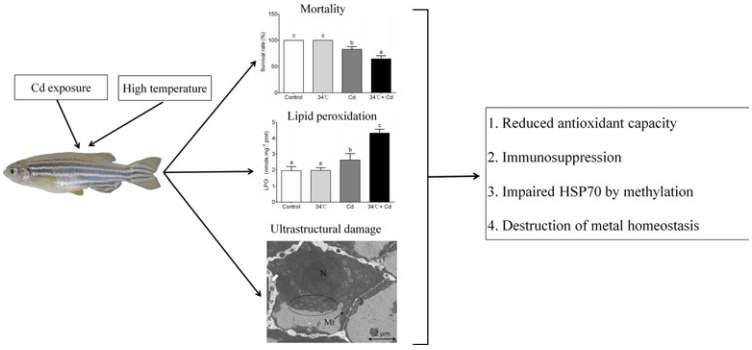
HIGHLIGHTS

- Warming increased Cd toxicity in fish.
- Mortality, lipid peroxidation and ultrastructure were examined.
- Activity, transcription and methylation of stress-related genes were analyzed.
- Sensitivity to warming varied between normal and Cd-exposed fish.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, female zebrafish (*Danio rerio*) were exposed to 26 °C or 34 °C, 0 or 197 μg/L cadmium (Cd), singly or in combination for 7 days. Multiple stress-related indicators were evaluated in the liver. Mortality, lipid peroxidation (LPO) and ultrastructural damage increased significantly by Cd exposure alone, and were not affected by heat alone. Interestingly, the combined exposure increased LPO, ultrastructural damage, and mortality compared with Cd exposure alone. The results indicated that elevated temperature increased Cd toxicity, which could be explained by several reasons. Firstly, Cd-exposed fish failed to activate the antioxidant defense system under heat stress. Secondly, expression levels of heat shock protein 70 (HSP70) were not significantly up-regulated by heat in Cd-exposed fish but increased by 117 times in Cd-free fish. Besides, hypermethylation of heat shock factor (HSF) binding motif in HSP70 promoter was observed during the combined exposure, indicating that simultaneous exposure may have partially suppressed the cytoprotective up-regulation of HSP70. Thirdly, heat induced an immunosuppressive effect in Cd-exposed fish, as reflected by the reduced mRNA and activity levels of nitric oxide synthase (iNOS) and interleukin-1β (IL-1β) expression levels. Finally, heat down-regulated Zir-, Irt-like protein 8 (ZIP8) and copper transporter 1 (CTR1) and up-regulated metallothioneins (MTs) in Cd-exposed fish, possibly suggesting Cu and Zn depletion and Cd accumulation. Hence, our data provide evidences that warmer temperatures can potentiate Cd toxicity, involved in the regulation of gene transcription, enzymatic activity, and DNA methylation. We found that heat indicators showed varied sensitivity between normal and Cd-exposed fish, emphasizing that the field metal pollution should be carefully considered when evaluating effects of climate change.

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1. Introduction

Anthropogenic derived impacts have unequivocally contributed to environmental poisoning by metals in aquatic ecosystems during the last few decades. Among metals, cadmium (Cd) is of particular concern as it is ubiquitous in the environment and highly toxic (Goering et al., 1995). Cd concentrations vary between 1.120 and 8 μg/L in rivers and lakes (He and Chen, 2007; Li and Liu, 2009) and even exceed 0.8 mg/L in some lakes in industrialized areas in China (Dong et al., 2015), which are much higher than those in normal freshwater (<0.5 μg/L). Thus, aquatic organisms are continuously threatened by Cd exposure.

The cumulative emissions of greenhouse gases have contributed to warming at a global scale (Olabisi et al., 2015). According to IPCC (2013) projections, the global mean surface temperature is expected to increase 6 °C by the end of this century as a result of global warming. Intensification of extreme events is emerging as another important aspect of global climate change (Jentsch et al., 2007). Heat waves have increased in frequency and severity in several regions of the world (Jentsch et al., 2007). Increases in air temperature will result in increased temperature in water, particularly in freshwater ecosystems (Kaushal et al., 2010). The increase of temperature in aquatic ecosystems is known to impact physiological processes in fish (Breckels and Neff, 2013).

In a natural environment, Cd exposure is not a single stressor, heat stress may occur simultaneously. The combined effects of temperature and Cd exposure have received more attention in aquatic organisms on lethal response (Piazza et al., 2016; Roch and Maly, 1979), histological alteration (Boughammoura et al., 2013), mitochondrial function (Onukwufor et al., 2015; Onukwufor et al., 2016), antioxidant response (Fadhlaoui and Couture, 2016; Vergauwen et al., 2013), and stress protein response (Ivanina et al., 2009; Yang et al., 2012; Olsson et al., 1996). All these studies suggest that elevated temperature increases Cd toxicity. However, information is still largely unknown on the role of immune response, metal transport, and gene epigenetic modification in this process.

In the immune system, inducible nitric oxide synthase (iNOS) is an early response gene in the inflammatory process and are commonly used as inflammatory markers (Ramyaa et al., 2013). Proinflammatory cytokines including interleukin-6 (IL-6) and interleukin-1β (IL-1β), act as modulators of immune responses, and are functionally active in immune response in teleosts (Bo et al., 2015; Grayfer et al., 2008; Karan et al., 2016; Zhang et al., 2016). In metal transport system, Zir-, Irt-like protein 8 (ZIP8) and copper transporter 1 (CTR1) are responsible for zinc and copper uptake. Metallothioneins (MTs) are involved in both transport and detoxification of metals, as well as scavenging of ROS (Hart et al., 2001). Regarding gene epigenetic modification, bioinformatics analysis indicates that heat shock elements (HSEs) are located in CpG Island of heat shock protein 70 (HSP70) promoter and have four methylation CpG loci in zebrafish. Heat shock protein 70 (HSP70) prevents protein denaturation and holds in a state of folding or assembly to facilitate repair (Izagirre et al., 2014; Yokoyama et al., 2000), which is transcriptionally controlled by heat shock factors (HSFs) (Ostling et al., 2007). Multiple defense systems are dependent on distinct mechanisms that modulate either catalytic activity of pre-existing enzymes, gene transcription, or DNA modification. Antioxidant defense mechanism has well elucidated in fish by detecting mRNA and activity levels of catalase (CAT) and copper/zinc-superoxide dismutase (Cu/Zn-SOD) during the combined exposure to Cd and heat (Fadhlaoui and Couture, 2016; Vergauwen et al., 2013). However, whether HSP70 methylation will change during the combined exposure to Cd and heat remains unclear.

Zebrafish (*Danio rerio*) have become an important vertebrate model organism and are recommended for toxicity testing (OECD guideline 203, 1992). Transcriptomic analysis suggests that zebrafish acclimation to 34 °C affects transcriptional levels of mounting genes including RNA processing, cellular metal ion homeostasis, cytokine signaling, and protein folding and transport (Long et al., 2012). The present study investigated several potential mechanisms that heat increases Cd toxicity. Cd toxicity was evaluated by determining survival rate and hepatic lipid peroxidation (LPO), and observing ultrastructural alteration. To uncover associated molecular mechanisms, different pathways including antioxidant defenses, immunity, metal homeostasis and protein folding were examined in the liver of zebrafish. The detected indicators included mRNA and activity levels of Cu/Zn-SOD, CAT and iNOS, and gene transcriptional levels of Cu/Zn-SOD, CAT, iNOS, MTs, IL-6, IL-1β, CTR1, ZIP8, and HSP70. Furthermore, the methylation profiles in the HSF binding motif of the HSP70 promoter were analyzed in the liver of zebrafish.

2. Materials and methods

2.1. Fish maintenance and treatment protocol

Adult zebrafish (AB strain) were maintained in charcoal-filtered and aerated tap water at 26 °C with a photoperiod of 12L: 12D for 2 weeks. At the beginning of the trial, uniform-sized fish (initial body weight: 0.41 ± 0.04 g, mean ± SEM) were divided into four groups and transferred in separate 30-L glass aquariums. Fish were maintained in Cd-free water at 26 °C to serve as control group, and the three other groups were exposed either to Cd (200 μg/L, corresponding to 1.78 μM) and/or to the high temperature of 34 °C for 7 days. Individual test solutions in the experiment were obtained by adding the appropriate volume of the primary stock to the dilution water. Water was renewed 100% at 7 am every morning, Cd was added as Cd chloride at the time of the water change when Cd concentrations were detected. The fish were fed commercial diets three times daily at a rate of 1% of body weight (10.6% lipid and 42.4% protein on a dry matter basis). Dissolved oxygen and pH were 7.42 ± 0.24 mg/L and 7.49 ± 0.33, respectively. Cd concentrations in Cd-free and Cd-exposed water were 0 and 197.4 ± 5.9 μg/L, respectively. The metal concentrations were measured using flame atomic absorption spectroscopy (FAAS) (Zheng et al., 2015).

At the end of the 7-day period, 24 h after the last feeding, fish were counted. All fish were euthanized by a 0.02% tricaine methanesulfonate solution (MS-222). Liver tissues from female zebrafish were separated, frozen in liquid nitrogen immediately, and stored at −80 °C till RNA extraction and biochemical detection. The other fresh samples were fixed for ultrastructural observations. We ensured that all protocols with live fish adhered the Zhejiang Ocean University's ethical instructions for the care and use of lab animals.

2.2. Ultrastructural observation

The ultrastructural observations were conducted according to the methods from Zheng et al. (2016a). Generally, specimens of liver were prefixed by immersion in 2.5% glutaraldehyde in phosphate buffer solution (pH 7.2) at 4 °C. Post-fixation was in cold 1% aqueous osmium tetroxide for 1 h. They were rinsed again in phosphate buffer, dehydrated in a graded ethanol series, and embedded in epon-araldite mixture. Ultrathin sections were cut and stained with uranyl acetate and lead citrate, and examined under a HITACHI H-600 electron microscope.

2.3. Biochemical analysis

Preparation and extraction of homogenates and supernatants were performed according to our previous study (Zheng et al., 2016b). The supernatants were maintained at 4 °C until being used for biochemical analysis. Lipid peroxidation (LPO) was determined by the thiobarbituric reactive species (TBARS) assay, which measured the production of malondialdehyde that reacts with thiobarbituric acid, according to the method described by Livingstone et al. (1990). Cu/Zn-SOD activity was measured according to the method of Beauchamp and Fridovich (1971) based on aerobic reduction of NBT at 535 nm by superoxide

radicals. CAT activity was determined by measuring the rate of disappearance of H_2O_2 at 240 nm according to methods described by Beutler (1982). Soluble protein content was determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. All enzyme activities were detected at 26 °C, expressed as U (units) per mg of soluble protein. All protein levels were expressed as ng per mg of soluble protein. Four biological replicates and two technical replicates were used in the analyses.

2.4. Expression levels of mRNA

RNA extraction, cDNA synthesis, and quantitative real-time PCR were carried out using RNAiso Plus Kit, PrimeScript® RT reagent Kit, and SYBR® Premix Ex Taq™ Kit according to manufacturer instructions of Takara, respectively. Each reaction was verified to contain a single product of the correct size using agarose gel electrophoresis and have no significant differences in amplification efficiencies (primers in Table 1). Abundances of transcripts of genes were calculated according to the $2^{-\Delta\Delta Ct}$ method, normalizing to the geometric mean of the best combination of β-actin and GAPDH (Zheng et al., 2016c).

2.5. Methylation analysis of HSP70

Genomic DNA was extracted from liver samples by using E.Z.N.A.® Tissue DNA Kit (Omega). DNA integrity was detected with 1% agarose gel electrophoresis, and the concentration was measured by a NanoDrop 2000c UV–vis Spectrophotometer (Thermo Fisher Scientific). 1 μg of DNA was bisulfite-modified using the EZ DNA Methylation-Gold Kit (Zymo Research) according to the manufacturer's instruction. The sequences of promoter and exon 1 of HSP70 (Ensembl: ENSDARG00000021924) were obtained and submitted to the online software Methprimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) to acquire the distribution of CpG islands and candidate CpG loci. Binding transcription factor HSF was predicted by MatInspector software (http://www.genomatix.de/cgi-bin/matinspector/matinspector). Primers were designed according to the known sequences using Oligo 6.0 to amplify target fragment (forward primer TTAATGATAGAATGAAAGATTATTAG GT; reverse primer: TTTTAATTTTCACCTTACCTTTTATAT). The resulting target fragments were purified using the Agarose Gel DNA Fragment Recovery Kit Ver.2.0 (Takara), subcloned using the PMD-19T Easy Vector System (Takara). The plasmid was used for transformation of JM109 Competent Cells (Promega, Nepean, Canada) and clones with inserts were sequenced (Sangon, Shanghai, China), 8 fish for one treatment with each fish 1–3 clones. The percentage of the number of converted cytosines was evaluated and reached 92.3%.

Table 1
Primers used for real-time PCR analysis from zebrafish.

Gene	Primer sequences (from 5′ to 3′)	Size (bp)	Accession no.
Cu/Zn-SOD	F: GTCCGCACTTCAACCCTCA R: TCCTCATTGCCACCCTTCC	227	BC055516
CAT	F: CAAGGTCTGGTCCCATAAA R: TGACTGGTAGTTGGAGGTAA	217	BC051626
MT	F: GCCAAGACTGGAACTTGCAAC R: CGCAGCCAGAGGCACACT	130	NM_131075
HSP70	F: GTCAGTGATGGAGGAAAGCCAA R: TGTGATAACTGCGTTGGTCACCT	153	AF210640
iNOS	F: CCTCCTCATGTACCTGAATCTCG R: GCTCCTGCTTTAGTATGTCGC	148	NM_001104937
IL-6	F: TCAACTTCTCCAGCGTGATG R: TCTTTCCCTCTTTTCCTCCTG	73	JN698962
IL-1β	F: TTCCCCAAGTGCTGCTTATT R: AAGTTAAAACCGCTGTGGTCA	149	NM_212844
ZIP8	F: TCCCCGCCTGCCCTTACACTT R: AGTGTCCCGATGGCCAGTCCAA	199	ENSDARG00000028214
CTR1	F: GGCCACGGAGATCACATGAT R: CTCCACATTTTTGTAGCCGAAGT	63	NM_205717
β-Actin	F: CTTGGGTATGGAATCTTGCG R: AGCATTTGCGGTGGACGAT	88	AF057040
GAPDH	F: GTAACTCCGCAGAAAAGCC R: CAAAAGAAACTAACACACAC	153	BC095386

2.6. Statistical analysis

Results are presented as mean ± standard error of means (SEM). Prior to statistical analysis, all data were tested for normality of distribution using the Kolmogorov–Smirnov test. The data were subjected to two-way ANOVA (heat and Cd exposure as factors) and Tukey's multiple range test. Analysis was performed using SPSS 18.0 for Windows (SPSS, Michigan Avenue, Chicago, IL, USA).

3. Results

3.1. Changes in ultrastructure

Ultrastructural alterations were evident in the single Cd exposure group, especially in the Cd exposure group in combination with heat (Fig. 1). In the control and heat groups, the hepatocytes were characterized by normal mitochondria with clear cristae and abundant endoplasmic reticulum (Fig. 1A, B). In the single Cd exposure group, there were largened intercellular space and cluster formation of mitochondria with clear cristae (Fig. 1C). In the combined exposure group, intercellular space was larger (Fig. 1D). The swelling, vesiculation, and cluster formation of mitochondria with disappeared cristae and absence of endoplasmic reticula were found.

3.2. Survival and LPO

Survival rate was significantly reduced by Cd exposure alone or in combination with heat (Fig. 2A). Heat decreased survival rate in Cd-exposed fish, and did not affect it in normal fish. There was a significant increase in LPO levels in the single Cd exposure group or the combined exposure group (Fig. 2B). LPO levels were increased by heat in Cd-exposed fish, and were not significantly affected in normal fish.

3.3. Enzyme activity

Cu/Zn-SOD activity was not affected by heat or Cd, singly or in combination (Fig. 3A). Cd exposure or heat stress alone did not significantly change CAT activity (Fig. 3B). However, the combined exposure to Cd and heat reduced CAT activity. iNOS activity increased by Cd or heat singly, but was not affected significantly by the combined exposure (Fig. 3C).

3.4. Gene expression

Expression levels of Cu/Zn-SOD, CAT, IL-6, and CTR1 were reduced by Cd exposure alone or the combined exposure (Fig. 4). The combined exposure further decreased expression levels of Cu/Zn-SOD and CTR1 compared with Cd exposure alone. Cd exposure alone sharply up-regulated mRNA levels of iNOS and IL-1β. However, the combined exposure reduced their transcriptional levels in Cd-exposed fish. Cd or heat exposure alone did not significantly affect ZIP8 expression levels, nonetheless, the combined exposure down-regulated ZIP8 expression. Heat stress increased HSP70 mRNA levels by 117 times in normal fish, while heat stress did not affect HSP70 mRNA levels in Cd-exposed fish. Heat also up-regulated MTs mRNA levels by 4 times in normal fish, and by 7 times in Cd-exposed fish.

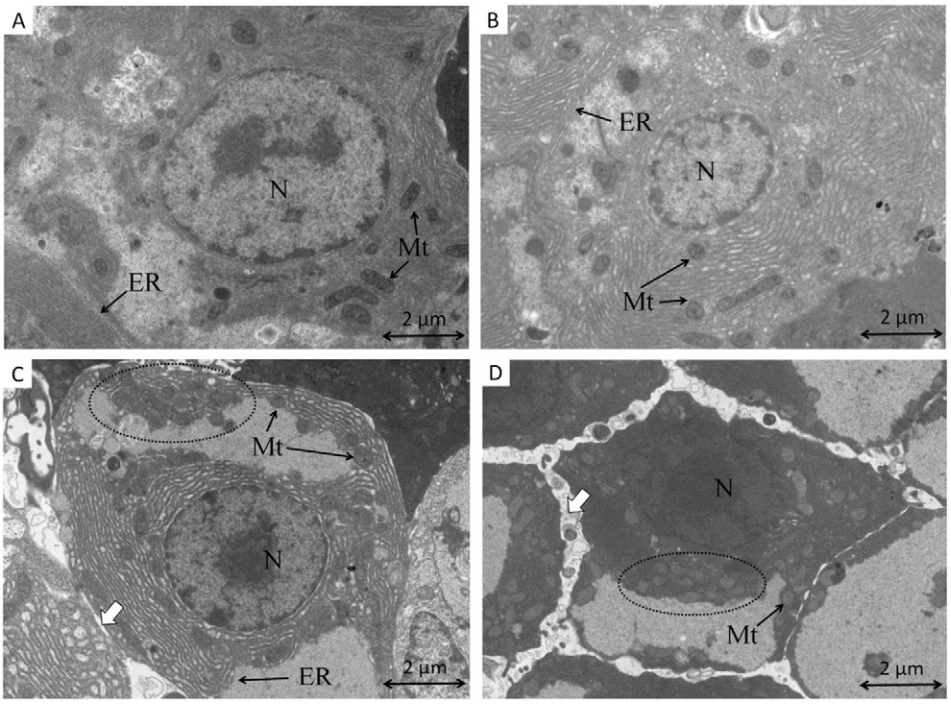

Fig. 1. Ultrastructure of hepatocytes of control fish (A), fish exposed to 34 °C (B), contaminated with 197 μg Cd/L (C), and co-treated with 34 °C and 197 μg Cd/L for one week (D). A and B: the normal structure of the hepatocyte, showing mitochondria with clear cristae. C: enlarged intercellular space (hollow arrow) and cluster formation of mitochondria with clear cristae (dotted circle) are found. D: intercellular space becomes larger, showing the swelling, vesiculation, and cluster formation of mitochondria with disappeared cristae and absence of endoplasmic reticula. N, nucleus; Mt, mitochondrial; ER, endoplasmic reticula.

3.5. Methylation of HSP70

The predicted CpG Island in HSP70 was 132 bp including part promoter and exon 1 sequences (Fig. 5A). One heat shock element was predicted between −80 and −59, which showed four CpG sites at −80, −78, −74, and −61. The methylation levels of these CpG sites are shown in Fig. 5B. Heat alone reduced methylation levels of CpG sites at −80, −78, and −61 compared to that of the control. Cd exposure alone reduced methylation levels of CpG sites at −80 and −74 but increased methylation levels of CpG sites at −78 and −61. The combined exposure increased methylation levels of CpG sites at −78, −74, and −61.

4. Discussion

The molecular mechanisms of interaction between heat and metal exposure are not yet clear. Before examining their interaction, we discuss the effects of high temperature and Cd exposure separately to facilitate our understanding of that why elevated temperature increases metal toxicity in fish.

4.1. Effects of heat stress

Regarding ectotherms, elevated temperature may represent a pro-oxidant stressor, as a common consequence of the metabolic-base disturbance (Matoo et al., 2013). Increasing environmental temperature leads to an increase in metabolic rate and thus to an increase in the production of reactive oxygen species (ROS) (Tesch et al., 2001), especially because mitochondria exposed to high temperatures are less efficient (Abele et al., 2002; Onukwufor et al., 2015; Sappal et al., 2015). Several studies have reported that acute exposure to elevated temperatures results in an increase in oxidative damage in fish (Cheng et al., 2015; Madeira et al., 2013; Vinagre et al., 2012). However, LPO levels were not significantly affected by high temperature in the present study. First of all, it is important to note that the lack of a significant effect of temperature on oxidative damage could be due to exposure time (Vinagre et al., 2012), sampling tissue (Vinagre et al., 2014), exposure

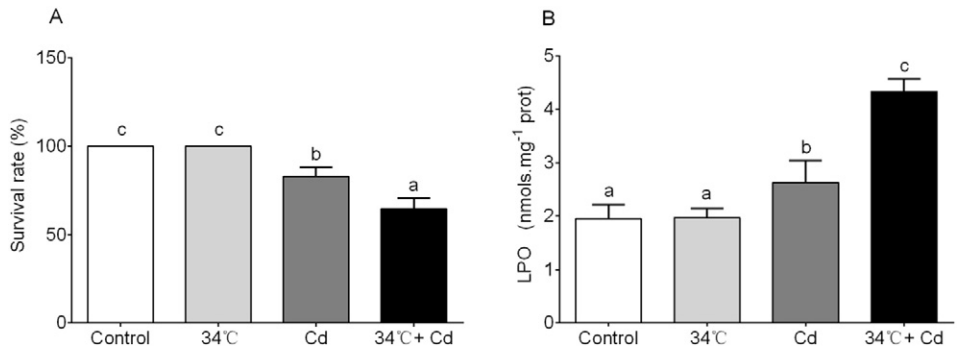

Fig. 2. Survival rate and hepatic LPO levels of control, fish exposed to 34 °C, contaminated with 197 μg Cd/L, and co-treated with 34 °C and 200 μg Cd/L for one week. Values are means ± SEM, n = 4. The different letters indicate significant difference among different treatments ($P < 0.05$).

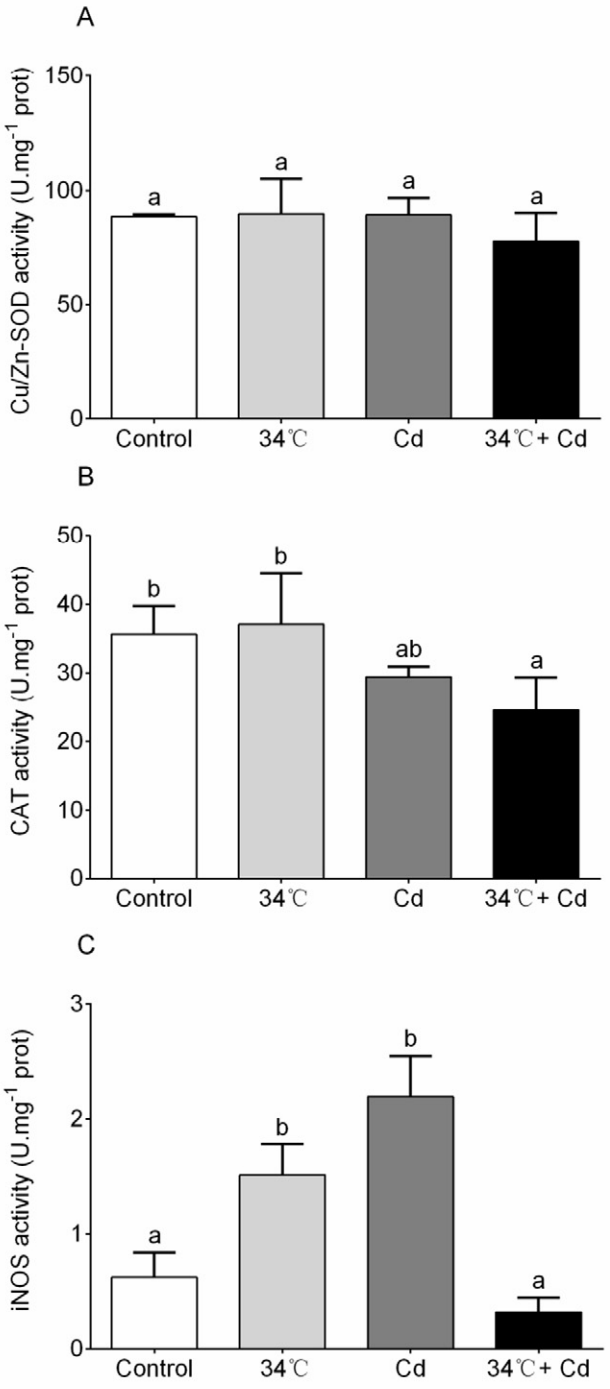

Fig. 3. Activities of Cu/Zn-SOD (A), CAT (B), and iNOS (C) in the liver of control, fish exposed to 34 °C, contaminated with 200 μg Cd/L, and co-treated with 34 °C and 197 μg Cd/L for one week. Values are means ± SEM, n = 4. The different letters indicate significant difference among different treatments ($P < 0.05$).

temperature (Dalvi et al., 2017; Vinagre et al., 2012), and water environment such as dissolved oxygen and salinity (Hu et al., 2015; Olsvik et al., 2013). In addition, the absence of observed differences could be related to fish species (Machado et al., 2014). Although zebrafish are mostly kept at 25–28 °C, they can adapt a wide range of environmental temperatures from as low as 6 °C in winter to over 38 °C in summer (Spence et al., 2008). Our results showed that the survival rate of heat group was 100% and no significant changes in structure of subcellular organelles, further confirming that zebrafish have a high tolerance to high temperature. Besides, activity levels of Cu/Zn-SOD and CAT remained relatively stable, indicating that heat did not induce oxidative stress.

Possibly, oxidative stress was scavenged by up-regulation of HSP70 and MTs. HSP70, a well-known component of the cellular heat shock response, protects cells from oxidative stress that typically results in protein misfolding (Cummings et al., 2001; Izagirre et al., 2014). MT acts as a radical scavenger to protect cells against the toxic effects of oxidative stress (Buico et al., 2008). Similar results that high temperatures induce an increase in HSP70 and MTs expression have been found in aquatic animals (Cheng et al., 2015; Cui et al., 2013; Duan et al., 2017; Ivanina et al., 2013; Keller et al., 2008; Maulvault et al., 2017; Long et al., 2012). Overexpression of HSP70 and MTs during the heat shock may result from a direct activation of the promoter by heat shock factor (HSF) (Ostling et al., 2007; Tamai et al., 1994). In the present study, heat reduced methylation levels of the HSF binding motif in the promoter of HSP70, suggesting the increase in HSP70 expression by heat might be attributed to hypomethylation of the HSF motif. iNOS is an early response gene in the inflammatory process and is commonly used as an inflammatory marker (Ramyaa et al., 2013). In the present study, the mRNA and activity levels of iNOS were significantly up-regulated by heat, indicating that heat induced inflammatory process (Polinski et al., 2013; Yang et al., 2016). The rapid induction of these genes may be to regulate the metabolism of cells during their adaptation to environmental changes for survival.

4.2. Effects of Cd exposure

The discussion of effects of Cd exposure will be considered at the optimal temperature of 26°Cto make a clear separation of the experimental factors. In the present study, increases in mortality and LPO, and changes in ultrastructure were found, reinforcing Cd toxicity in fish (Guo et al., 2017; Yuan et al., 2017; Zheng et al., 2016a, 2016b, 2016c). The negative effects of Cd exposure may be explained by several reasons. Firstly, CAT activity decreased by 17% ($P > 0.05$) and the mRNA levels of Cu/Zn-SOD and CAT were significantly reduced by Cd, which resulted in an increased risk of oxidative damage. Secondly, cytokine IL-6 expression declined, reflecting that Cd exhibited their negative effects on the immune system response. The decrease in mRNA levels of IL-6 indicated a suppressive effect on immunity by Cd, similar to other studies on fish (Giri et al., 2016; Guardiola et al., 2013; Guo et al., 2017). Thirdly, a failure to initiate activation of HSP70 and MTs may contribute to an increase in misfolded proteins and Cd^{2+} accumulation respectively, as reflected by no significant increase in demethylation of HSF motif and mRNA levels of both genes. Finally, CTR1 and ZIP8 are responsible for the cellular uptake of Cu and Zn, respectively (Feeney et al., 2005; Leung et al., 2014). The result of our study showed a decreased CTR1 expression after Cd exposure, possibly suggesting a reduction of the essential trace element Cu. Although Cd exposure exhibited its toxicity via these aforementioned mechanisms, Cd up-regulated IL-1β and iNOS and did not significantly decrease Cu/Zn-SOD activity and ZIP8 expression, which may partially compensate negative effects of Cd.

4.3. Combined effects of Cd exposure and heat stress

Based on survival rate observations, Cd toxicity clearly increased with elevated temperature. Similarly, the combined exposure induced severe alterations of hepatocellular structure and increased LPO levels compared with the control or Cd exposure alone. The result indicated that heat increased Cd toxicity, in accordance with other studies on fish (Fadhlaoui and Couture, 2016; Hallare et al., 2005; Onukwufor et al., 2015; Sassi et al., 2010; Vergauwen et al., 2013). This may be due to the fact that the high temperature tends to increase the diffusion, uptake and reaction rates of metals (Bervoets et al., 1996; Reynolds and Casterlin, 1980), thereby increasing the toxic action of Cd. Cd occupies transport systems of Zn and Cu, and competes with them (Dalton et al., 2005; Komjarova and Bury, 2014). High temperature increases

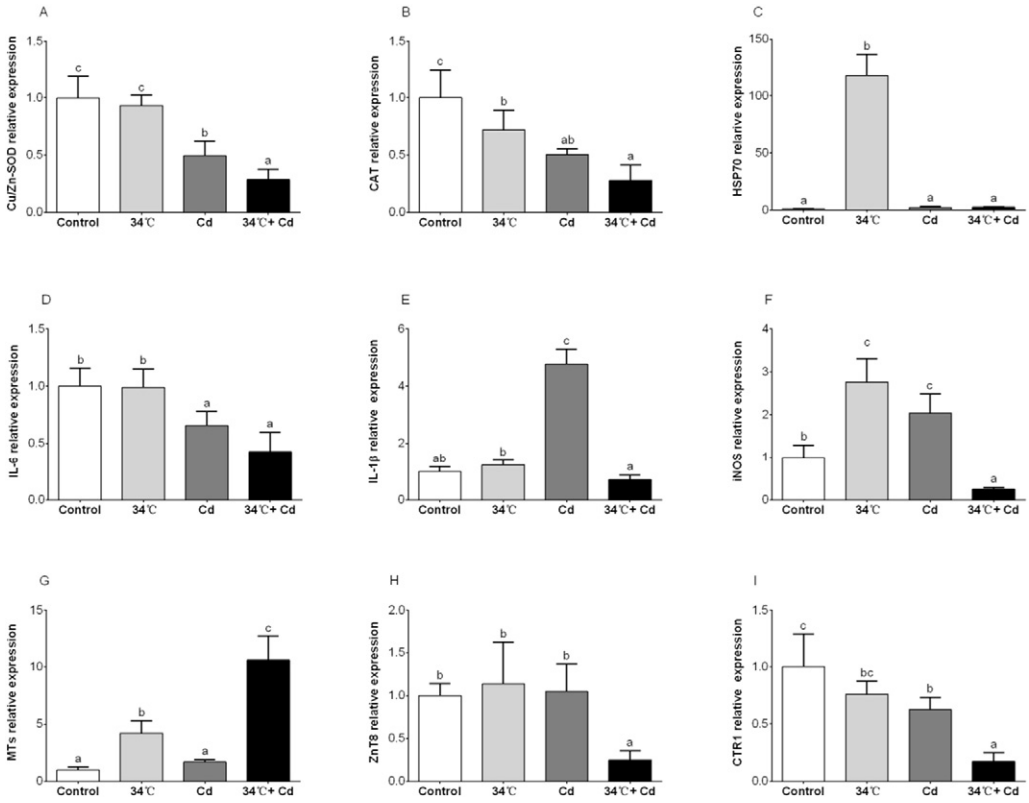

Fig. 4. Relative expression levels of genes in the liver of control, fish exposed to 34 °C, contaminated with 197 μg Cd/L, and co-treated with 34 °C and 200 μg Cd/L for one week. Values are means ± SEM, n = 4. The different letters indicate significant difference among different treatments ($P < 0.05$).

Cd accumulation (Vergauwen et al., 2013). The result of our study showed the reduced mRNA levels of CTR1 and ZIP8, which might be a vital compensatory mechanism by which fish slow the uptake of Cd, Zn and Cu. In the present study, heat increased MTs expression by 7 times in fish exposed to Cd, contributing to mitigating Cd impacts. However, high MT levels could not fully prevent disturbances to the cellular redox status during this combined exposure, as reflected by down-regulation of antioxidant genes and increased LPO levels.

In the present study, heat stress increased HSP70 mRNA levels by 117 times in normal fish, while heat stress did not affect HSP70 mRNA levels in Cd-exposed fish. Since it is unlikely that Cd exposure relieves the degree of protein damage thus counteracting the effects of heat stress, the explanation of this decrease in HSP70 levels is an impaired ability of HSP70 synthesis during the combined exposure to heat and Cd. Pytharopoulou et al. (2006) suggested Cd impacted cellular biosynthetic capacity such as transcription and/or translation processes. We found hypermethylation of HSF motif in the promoter of HSP70, which may result in a failure to activate heat shock response. In the present study, the combined exposure significantly down-regulated expression levels of IL-1β and iNOS and activity levels of iNOS compared with the single Cd exposure, indicating that heat stress caused an immunocompromised effect in fish exposed to Cd. Pro-inflammatory

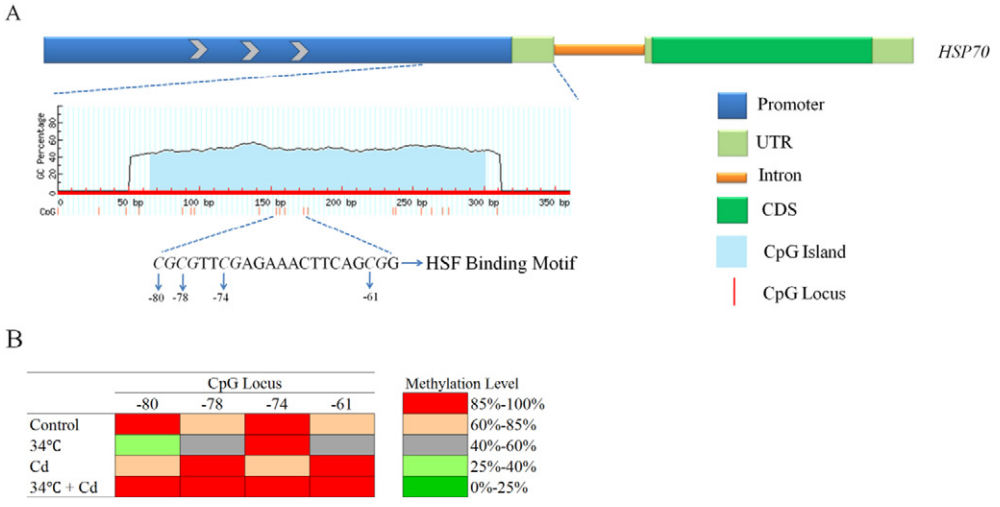

Fig. 5. The methylation profiles in HSF binding motif of HSP70 in the liver of control, fish exposed to 34 °C, contaminated with 197 μg Cd/L, and co-treated with 34 °C and 197 μg Cd/L for one week. (A) Gene structure of HSP70, showing the distribution of CG Island and candidate CpG sites in HSF binding motif. (B) Methylation percentage of HSP70 at 4 single CpG sites in HSF binding motif in promoter.

cytokine IL-1β stimulates immune responses by activating lymphocytes or inducing the release of other cytokines that then activate macrophages, NK cells, and lymphocytes (Low et al., 2003). The down-regulation of IL-1β may result in reduced phagocytosis during the combined exposure (Giri et al., 2016; Guardiola et al., 2013). The iNOS/NO pathway is known to play vital roles in maintaining innate immunity and acquired immunity (Bogdan, 2001). Impaired bioactivity of NO and iNOS is implicated in major risk factors for atherosclerotic vascular disease (Böger et al., 1995; Pi et al., 2003), and embryopathy in mammals (Nath et al., 2004; Veeriah et al., 2015).

5. Conclusion

The present work demonstrated that elevated temperature increased Cd toxicity. Indicators of mortality, oxidative damage, and histopathology supported direct evidences. On molecular and enzymatic levels, heat stress alone up-regulated MTs, HSP70, and iNOS and did not affect antioxidant and metal transport systems, contributing to neutralizing potential negative effects. Although Cd exposure alone stimulated inflammatory response, a failure to activate HSP70 and MTs, the down-regulation of antioxidant defense, and the reduced uptake of Cu may result in an increased Cd toxicity. Cd-induced impairment was more severe when fish were subjected to high temperature. Serious injuries were observed, involved in immunity, antioxidant defenses, heat shock response, uptake of essential metals such as Cu and Zn.

To sum up, the present work provided preliminary insights on the possible impacts of global warming and frequent heat waves, particularly in freshwater ecosystem that subjected to strong anthropogenic pressure and high levels of chemical contaminants, such as Cd. Furthermore, most biomarkers except for HSP70, MT, and iNOS appeared to be poor indicators of heat stress in normal fish, but were greatly affected by heat in Cd-contaminated fish. Hence, we suggest that the field metal pollution to which fish are being exposed should be carefully considered when evaluating the impacts of climate change on aquatic organisms.

Acknowledgment

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